Transforming Growth Factor-β1 Regulates Axon/Schwann Cell Interactions

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Abstract. We have investigated the potential regulatory role of TGF-β in the interactions of neurons and Schwann cells using an in vitro myelinating system. Purified populations of neurons and Schwann cells, grown alone or in coculture, secrete readily detectable levels of the three mammalian isoforms of TGF-β; in each case, virtually all of the TGF-β activity detected is latent. Expression of TGF-β1, a major isoform produced by Schwann cells, is specifically and significantly downregulated as a result of axon/Schwann cell interactions. Treatment of Schwann cells or Schwann cell/neuron cocultures with TGF-β1, in turn, has dramatic effects on proliferation and differentiation. In the case of purified Schwann cells, treatment with TGF-β1 increases their proliferation, and it promotes a pre- or nonmyelinating Schwann cell phenotype characterized by increased NCAM expression, decreased NGF receptor expression, inhibition of the forskolin-mediated induction of the myelin protein P0, and induction of the Schwann cell transcription factor suppressed cAMP-inducible POU protein. Addition of TGF-β1 to the cocultures inhibits many of the effects of the axon on Schwann cells, antagonizing the proliferation induced by contact with neurons, and, strikingly, blocking myelination. Ultrastructural analysis of the treated cultures confirmed the complete inhibition of myelination and revealed only rudimentary ensheathment of axons. Associated defects of the Schwann cell basal lamina and reduced expression of laminin were also detected. These effects of TGF-β1 on Schwann cell differentiation are likely to be direct effects on the Schwann cells themselves which express high levels of TGF-β1 receptors when cocultured with neurons. The regulated expression of TGF-β1 and its effects on Schwann cells suggest that it may be an important autocrine and paracrine mediator of neuron/Schwann cell interactions. During development, TGF-β1 could serve as an inhibitor of Schwann cell proliferation and myelination, whereas after peripheral nerve injury, it may promote the transition of Schwann cells to a proliferating, nonmyelinating phenotype, and thereby enhance the regenerative response.

PERIPHERAL nerve development progresses through a series of distinct stages that reflect complex and reciprocal interactions between axons and Schwann cells (Webster, 1992). Initially, nerve fibers grow out essentially free of nonneuronal cells. Subsequently, Schwann cells migrate and proliferate on the nerve fibers, progressively subdividing the nerve fascicle into smaller groups of nerve fibers. Eventually, Schwann cells either communally ensheathe multiple small nerve fibers, or they myelinate individual nerve fibers with which they have established a one-to-one relationship (Webster, 1992). In both instances, the axon/Schwann cell unit is surrounded by a basal lamina that is principally synthesized by Schwann cells when they are in contact with neurons (Bunge et al., 1982). This basal lamina, in turn, is required for the appropriate function of the Schwann cell; defects in basal lamina production are associated with defects in the ensheathment and myelination of axons (Bunge et al., 1986).

The different anatomic relationships that Schwann cells establish with axons correspond to distinct differentiated phenotypes. Nonmyelinating, ensheathing Schwann cells express high levels of the adhesion molecules L1 and the neural cell adhesion molecule (NCAM)[1] do not express myelin proteins, and contain a distinct set of cytoskeletal proteins. By contrast, myelinating Schwann cells express low levels of NCAM and L1, but high levels of the myelin as-

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1. *Abbreviations used in this paper*: DRG, dorsal root ganglion; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; BrDU, 5-bromo-2'deoxyuridine; NCAM, neural cell adhesion molecule; SCIP, suppressed cAMP-inducible POU protein; MLEC, mink lung epithelial cells; PAI, plasminogen activator inhibitor.

sociated glycoprotein (MAG) and several structural components of the myelin sheath, including myelin basic protein (MBP) and P0 (reviewed in Jessen and Mirsky, 1991; Salzer, 1995). Like proliferation and basal lamina formation, the differentiation of Schwann cells is regulated by the axon as their ensheathment fate is specified by the type of axon with which they are associated (Aguayo et al., 1976; Weinberg and Spencer, 1976).

After injury, there are dramatic changes in peripheral nerves distal to the site of the lesion (reviewed in Fawcett and Keynes, 1990). Expression of myelin proteins rapidly declines (Lemke and Chao, 1988) and Schwann cells reexpress NCAM and L1 (Martini and Schachner, 1988). Schwann cells also undergo a wave of proliferation at the site of injury in all nerves and in the distal stump of heavily myelinated nerves (reviewed briefly in Salzer and Bunge, 1980). Concomitantly, macrophages invade the distal stump and, together with resident Schwann cells, they break down and clear the degenerating myelin (Stoll et al., 1989). Finally, if the nerve has not been permanently transected, new nerve fibers sprout from the proximal end of the injured nerve and grow into the distal stump guided by Schwann cells. The downregulation and clearance of myelin proteins and the reexpression of cell adhesion molecules by Schwann cells, together with their synthesis and release of neurotrophic factors, is thought to be critical for successful regeneration of peripheral nerves after injury (Scherer and Asbury, 1993).

The molecular signals that regulate Schwann cell proliferation and differentiation during peripheral nerve development and injury are not well understood. Molecules associated with the neuronal surface are known to induce Schwann cell proliferation during development (Salzer et al., 1980), and they are likely to correspond, at least in part, to the recently described family of neuregulins (Marchionni et al., 1993), also referred to as glial growth factor and heregulin (Peles and Yarden, 1993). It is not yet known whether the mitogenic signals that stimulate Schwann cell proliferation during nerve injury are related to those that operate during development. However, a recent study reporting that soluble mitogenic factors are released as a result of injury (Wen et al., 1994) suggests that they could be distinct. An additional potential source of mitogenic factors during nerve injury are macrophages, which invade the distal stump in high numbers and have been proposed to promote Schwann cell proliferation during injury (Beuche and Friede, 1984) via soluble factors (Baichwal et al., 1988). However, the proliferation of Schwann cells during Wallerian degeneration in vitro, in the absence of macrophages (Salzer and Bunge, 1980), suggests that nonmacrophage-derived mitogens, potentially growth factors released by the Schwann cells themselves, are also likely to be important.

Both proliferative and differentiative signals may be mediated via an increase in intracellular levels of cAMP. At low cell density, elevation of cAMP levels increases Schwann cell proliferation, whereas at high cell densities, particularly when proliferation is limited by growing cells in defined media without serum, an increase in cAMP leads to an increase in the expression of myelin proteins (Jessen and Mirsky, 1991; Morgan et al., 1991). In view of these findings, treatment of Schwann cells with the diterpene analogue forskolin, an activator of adenylate cyclase, has been used to mimic the effects of the axon on Schwann cells, particularly inducing the myelinating phenotype. Forskolin treatment, in addition to increasing expression of myelin proteins, also dramatically elevates the expression of the transcription factor SCIP (suppressed cAMP-inducible POU protein) by Schwann cells (Monuki et al., 1989). This protein, which was independently identified by several groups and is also called tst-1 (He et al., 1990) and Oct-6 (Suzuki et al., 1990), belongs to the POU domain family of transcription factors. It is prominently expressed by Schwann cells during periods of rapid proliferation, i.e., during development and transiently after peripheral nerve injury (Monuki et al., 1990; Scherer et al., 1994). Its expression in vivo has also been inversely correlated with the ability of Schwann cells to myelinate. Therefore, SCIP has been suggested to be a marker of, and may function in, proliferating, premyelinating Schwann cells. Consistent with this suggestion, SCIP inhibits the expression of myelin proteins, strongly repressing the P0 promoter, and also inhibiting the expression of the p75 NGF receptor (Monuki et al., 1990).

These studies suggest that there is an inverse relationship between Schwann cell proliferation and expression of the myelinating phenotype. Consistent with this notion, several Schwann cell mitogens, notably FGF and members of the TGF-β family (Ridley et al., 1989; Davis and Stroobant, 1990; Schubert, 1992), have been reported to inhibit the expression of myelin proteins induced by forskolin (Mews and Meyer, 1993; Rogister et al., 1993; Morgan et al., 1994). These results raise the possibility that these growth factors may have a role as inhibitors of Schwann cell myelination, although it is not known under what conditions and by which cells these growth factors are released. In contrast, axons may also release soluble factor(s) that promote the myelinating phenotype, transiently inducing the expression of SCIP, for example, and leading to a several-fold increase in P0 expression (Bolin and Shooter, 1993). These findings suggest that soluble mediators might function as both positive and negative regulators of Schwann cell myelination.

In this study, we have investigated the role of TGF-βs as mediators of axon/Schwann cell interactions. We focused on the TGF-βs because of their critical role in regulating cell growth and differentiation in many developing systems (Massagué, 1990; Sporn and Roberts, 1992) and their mitogenic effects on Schwann cells. The TGF-β family is comprised of three mammalian isoforms termed TGF-β1, -β2, and -β3, and the TGF-β superfamily contains, in addition, a large number of homologous proteins (see Kingsley, 1994, for a recent review). We have principally focused on the role of TGF-β1 in this study because, as we now report, TGF-β1 is a prominent isoform produced by Schwann cells, and the expression of TGF-β1 appears to be regulated by axon/Schwann cell interactions. We have found that TGF-β1, which is a mitogen for purified Schwann cells, inhibits the proliferation of Schwann cells that is normally induced by neuronal contact. In addition, TGF-β1 increases the expression of markers of premyelinating Schwann cells, notably NCAM and SCIP, and it inhibits the forskolin-induced transition to a myelinating phenotype. Consistent with these findings, TGF-β1 strikingly inhibits myelination in Schwann cell/neuron cocultures and leads to associated defects in the formation of the Schwann cell basal lamina. These results indicate that TGF-β1 has profound effects on the axonal induction of Schwann cell proliferation and differentiation, and they sug-

gest that it could be an important inhibitor of Schwann cell proliferation and myelination during development. These studies also suggest an important role for TGF-β1 in the regenerative response that follows Wallerian degeneration in the peripheral nervous system.

Materials and Methods

Antibodies and Growth Factors

Antibodies used in this study included anti-MBP and anti-P0 polyclonal antibodies (gifts from D. Colman, Mount Sinai Medical Center, NY), a polyclonal anti-p75 NGF receptor antibody (gift from Dr. B. Hempstead, Cornell University Medical Center, NY), monoclonal antibody MA513 to the MAG (gift from M. Schachner, Swiss Federal Institute of Technology, Hönggerberg, Zürich), anti-L1 polyclonal antibody (gift from M. Grumet, New York University Medical School, NY), anti-NCAM polyclonal antibody (gift from U. Rutishauser, Case Western Reserve University, Cleveland, OH), anti-SCIP polyclonal antibody (gift from M. G. Rosenfeld, University of California, San Diego, CA) and anti-laminin polyclonal antibody (Sigma Chemical Co., St. Louis, MO). Neutralizing antibodies against recombinant human TGF-β1, native porcine TGF-β2, and recombinant chicken TGF-β3 were purchased from R & D Systems, Inc. (Minneapolis, MN). A monoclonal antibody that neutralizes rat TGF-β1, TGF-β2, and TGF-β3 was purchased from Genzyme Corp. (Cambridge, MA). Species-specific, affinity-purified, rhodamine-conjugated donkey anti-rabbit IgG and fluorescein-conjugated donkey anti-mouse IgG were purchased from Chemicon International Inc. (Temecula, CA); fluorescein-conjugated mouse monoclonal antibodies directed against bromodeoxyuridine (BrDU) were obtained from Boehringer Mannheim Corp. (Indianapolis, IN).

Recombinant human TGF-β1 was a gift from Berlex Biosciences (South San Francisco, CA), recombinant human TGF-β2 was a gift from Celtrix (Santa Clara, CA), and TGF-β3 was purchased from R & D Systems. All concentrated stocks of TGF-β were stored at 4°C in a solution of 5 mM HCl containing 1 mg/ml low endotoxin BSA (ICN Biomedicals, Inc., Costa Mesa, CA).

Tissue Culture Methods

Cultures of primary rat Schwann cells, dorsal root ganglion (DRG) neurons, and myelinating Schwann cell/DRG neurons were established as described previously (Einheber et al., 1993). Briefly, cultures of dissociated rat embryonic day 16 DRG neurons were grown on collagen-coated 12-mm glass coverslips in a four-well dish (Nunc, Naperville, IL) and cycled with antimitotic agents in standard serum containing media to remove nonneuronal cells. The standard media consists of MEM (Whittaker Bioproducts, Inc., Walkersville, MD) supplemented with 10% FBS, 2 mM glutamine, 0.4% glucose, and 50 ng/ml 2.5S NGF (Bioproducts for Science, Inc., Indianapolis, IN). To establish myelinating cultures, DRG neuron cultures were seeded with 200,000 Schwann cells in standard media. Based on cell morphology, these Schwann cell preparations contained fewer than 0.1% fibroblasts. On the next day, the standard media was replaced with N2 defined media (5 mg/ml insulin, 10 mg/ml transferrin, 20 nM progesterone, 100 mM putrescine, 30 nM selenium, and 2 mM glutamine in a 1:1 mixture of DME and Ham's F-12 supplemented with 2.5S NGF). In this media, Schwann cells in contact with neurites proliferate in response to a neuronal mitogen, but they do not assemble a basal lamina or myelinate (Moya et al., 1980). The cultures were maintained in N2 media for 3 d to allow the Schwann cells to populate the neurites. To initiate basal lamina formation and myelination, the cultures were fed the standard media supplemented with 50 mg/ml ascorbic acid.

Determination of TGF-β Activity

Concentrations of total and active TGF-β present in serum-free culture supernatants were measured using the plasminogen activator inhibitor-1 promoter luciferase (PAI/L) assay as described (Abe et al., 1994). In this assay, serum-free culture conditioned supernatants are incubated with mink lung epithelial cells (MLEC) stably transfected with an expression construct containing a portion of the TGF-β-inducible plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reporter gene. Exposure of the transfected MLEC to TGF-β results in a dose-dependent increase in luciferase activity in lysates of the cells, as measured with a luminometer.

12 separate coverslips, each containing dissociated DRG neurons seeded with 200,000 Schwann cells, were maintained on N2 media for 3 d. Six of these cultures were switched to standard media (nonmyelinating cultures) and six to standard media supplemented with ascorbic acid (myelinating cultures). Parallel cultures of neurons alone and Schwann cells alone (400,000 Schwann cells/collagen coated coverslip) were maintained as described for the nonmyelinating cultures. (This number of Schwann cells represents an estimate of the minimum number of cells actually present in the cocultures based on cell counts from random fields). The cultures were fed their respective media every 2 or 3 d for a total of 8 d. The cultures were washed three times with N2 media, and they were incubated in 0.2 ml of N2 media for an additional 2 d. The conditioned N2 media from each group of cultures was collected, pooled, and spun 10 min at 4°C in a tabletop centrifuge to remove cellular debris. To determine the levels of total TGF-β (active and latent), conditioned media were heated for 12 min at 80°C to activate latent TGF-β. Active TGF-β levels were determined from unheated, undiluted conditioned media. Other samples were diluted to 20% with N2 media and added to the PAI/L-transfected MLEC in 96-well plates. To determine the total amount of PAI-1 promoter activity specifically induced by TGF-β in the samples, a neutralizing anti-TGF-β1,2,3 monoclonal antibody (20 μg/ml) was added to the diluted conditioned media and incubated with the transfected MLEC. The decrease in the amount of luciferase expressed in the presence of this antibody (~75% of the total luciferase in each case) was used to calculate the amount of active and total (active plus latent) TGF-β in the conditioned media. Similarly, the amount of PAI-1 promoter activity induced by the individual TGF-β isoforms was determined by the addition of TGF-β1, -β2, or -β3 neutralizing antibodies (20 μg/ml) to the assay. To generate standard curves of TGF-β isoform activity in the assay, serial dilutions of TGF-β1, -β2, or -β3 (1.5–800 pg/ml) in N2 media were incubated with the transfected MLEC. After an overnight incubation at 37°C in a 5% CO_2 incubator, the MLEC were washed with PBS and extracted with lysis buffer (Analytical Luminescence Laboratory, San Diego, CA). The cell lysates were analyzed for luciferase activity using luciferin substrate (Analytical Luminescence Laboratory) and a luminometer (ML1000; Dynatech Laboratories Inc., Chantilly, VA).

In parallel, we also performed a series of controls with each of the antibodies used in these assays to determine their specificity and efficiency in neutralizing each of the TGF-β isoforms. On average, the anti-TGF-β1,2,3 monoclonal antibody inhibited 79%, 89%, and 83% of purified TGF-β1, TGF-β2, and TGF-β3, respectively. The anti-TGF-β1 antibody inhibited 80%, 3%, and 5% of purified TGF-β1, TGF-β2, and TGF-β3, respectively; the anti-TGF-β2 antibody inhibited 3%, 92%, and 20% of purified TGF-β1, TGF-β2, and TGF-β3, respectively; and the anti-TGF-β3 antibody inhibited 6%, 39%, and 94% of purified TGF-β1, TGF-β2, and TGF-β3, respectively. (Thus, the anti-TGF-β1 antibody was highly specific; the antibodies to TGF-β2 and TGF-β3 displayed some cross-reactivity with recombinant TGF-β3 and TGF-β2, respectively). The calculated concentrations of TGF-β isoforms were corrected for this cross-reactivity.

Effects of TGF-β1 on the Expression of Schwann Cell Markers

Primary rat Schwann cells were expanded in tissue culture flasks (T-75 Primaria; Falcon, Oxnard, CA) or poly-L-lysine-coated flasks in D media (DME containing 10% FBS and 2 mM glutamine) supplemented with 2 μM forskolin and 10 μg/ml crude glial growth factor prepared as described (Porter et al., 1986). Once confluent, the cultures were maintained in D media for at least 7 d before addition of growth factors. To examine the effects of TGF-β1 and forskolin on Schwann cell protein expression, flasks of Schwann cells were fed with either N2 media or D media containing one of the following: 1 ng/ml TGF- β1, 10 ng/ml TGF-β1, 10 μM forskolin, or a combination of 10 ng/ml TGF-β1 and 10 μM forskolin for 7 d. Control cultures were fed the corresponding media containing an appropriate amount of TGF-β1 diluent. Cultures receiving N2 media or D media containing the added factors were fed either twice or three times, respectively, during the 7 d of treatment. In the N2 media experiments, three flasks were used for each treatment, and four flasks were maintained as controls. Only one flask was used per culture condition in the D media experiments.

Expression of Schwann cell markers was determined by immunoblot analysis of cell lysates. To prepare lysates, the cultures were washed with PBS, scraped into lysis buffer (95 mM NaCl, 25 mM Tris-Cl, pH 7.4, 10 mM EDTA, 2% SDS, 1 mM PMSF, and 10 mg/ml each of antipain, pepstatin A, and leupeptin), incubated in a boiling water bath for 5 min, and then briefly sonicated. The lysates were spun in a microfuge to remove insoluble material, and the protein concentrations of the cleared supernatants were

determined using the Micro BCA method (Pierce Chemical Co., Rockford, IL). 75 μg of protein from each lysate was fractionated on a 5–15% SDS polyacrylamide gel and blotted onto nitrocellulose. Blots were incubated with primary antibodies followed by ^{125}I-labeled protein A (Amersham Intl.) and exposed for autoradiography. Quantitation of the immunoreactive bands on the blots was performed on a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA).

Effects of TGF-β1 on Myelination

To examine the effects of TGF-β1 on the differentiation of Schwann cells, cocultures of Schwann cells and DRG neurons were grown under myelin-promoting conditions (standard media containing ascorbic acid) in the presence or absence of 1 or 10 ng/ml TGF-β1. TGF-β1 was added to cultures either when they were initially switched to myelin-promoting conditions or, alternatively, after they had been maintained in myelinating conditions for 2 d. The media for control cultures and cultures treated with 1 ng/ml TGF-β1 were supplemented with the TGF-β1 diluent (i.e., 1 mg/ml low endotoxin BSA in 5 mM HCl) equal in amount to that added to the cultures treated with 10 ng/ml TGF-β1. Cultures were subsequently given the standard media containing ascorbic acid with or without TGF-β1 every 2 or 3 d. After 8 d of growth under myelinating conditions, the cultures were processed for immunofluorescence or electron microscopy (described below). To determine the number of myelin segments present in the control and TGF-β1–treated cultures, the coverslips were immunostained for MBP and examined by epifluorescence on a microscope (Axiophot; Carl Zeiss, Inc., Thornwood, NY). A transparent grid was placed over the coverslips to facilitate counting the MBP-positive myelin segments on each coverslip. Statistical analyses were performed using the StatView program (Abacus Concepts, Inc., Berkeley, CA).

Effects of TGF-β1 on Schwann Cell Proliferation

The proliferation of Schwann cells grown in the absence or presence of neurons was determined using a BrDU nuclear labeling assay. To investigate the effects of TGF-β1 on Schwann cells grown alone, 100,000 Schwann cells were plated onto poly-L-lysine–coated glass coverslips in standard media. The next day, the cultures were fed standard media with or without 1 or 10 ng/ml TGF-β1; control cultures and cultures treated with 1 ng/ml TGF-β1 were also supplemented with TGF-β1 diluent equal in amount to that added to the cultures treated with 10 ng/ml TGF-β1. After ~72 h, the culture media was supplemented with BrDU (10 μM final concentration). The cultures were incubated with BrDU in a 7% CO_2 incubator at 35°C for 3.5 h. Cultures were washed in Dulbecco's PBS, fixed in 100% methanol at -20°C for 15 min, and incubated in 2 N HCl for 10–30 min. The HCl solution was removed and the cultures neutralized by two 5-min incubations in 0.15 M sodium borate buffer, pH 8.4, and several washes with L15 media (GIBCO BRL, Gaithersburg, MD). The cultures were then blocked with L15 media containing 10% serum for 30 min, and they were incubated with fluorescein-conjugated anti-BrDU antibody for 1 h at room temperature. Coverslips were washed in PBS and mounted on glass slides in Citifluor (Citifluor Ltd., London, U.K.) containing 1 mg/ml Hoechst dye.

The effects of TGF-β1 on the proliferation of Schwann cells in coculture with neurons were also investigated. In these studies, neuron cultures were seeded with 200,000 Schwann cells in standard media and maintained in N2 media for an additional 3 d. The cultures were then fed standard media containing ascorbic acid to promote myelination. TGF-β1 was added to cultures either when they were switched to myelin-promoting media or, alternatively, after they had been maintained in this media for 2 d. The BrDU proliferation assay was performed ~20 h after the addition of TGF-β1 to the cultures. Proliferation assays were also performed on cocultures maintained for 7 d in standard media after seeding neurons with Schwann cells. These cultures were switched to standard media containing ascorbic acid with or without TGF-β1 for 20 h before the proliferation assay was performed.

BrDU- and Hoechst dye–labeled nuclei in random fields of the coverslips were photographed using a Zeiss Axiophot microscope and slide film (Ektachrome 160T; Eastman Kodak Co., Rochester, NY). At least five or six random fields were photographed, typically using a 20× objective. The number of BrDU- and Hoechst dye–labeled nuclei in each field were then counted in a blinded manner from the Ektachrome slides projected on a slide viewer. In the case of Schwann cells grown alone, ~1,500 cells per condition were counted; in the case of the Schwann cell/neuron cocultures, in excess of 2,000 cells per condition were counted.

Immunofluorescence Microscopy

Cultures were processed for immunofluorescence microscopy as described previously (Einheber et al., 1993).

Electron Microscopy

Control and TGF-β1–treated myelinating cultures, grown on collagen-coated aclar plastic coverslips, were rinsed in PBS and fixed overnight at 4°C in 0.05M sodium phosphate buffer, pH 7.0, containing 2% glutaraldehyde and 0.1 M sucrose (Owens and Bunge, 1989). After washing in 0.1 M phosphate buffer, the coverslips were incubated in 2% osmium tetroxide in 0.1 M phosphate buffer for 1 h and embedded in Epon (Milner and Bacon, 1989). Portions of the coverslips were removed and reembedded in an orientation suitable for obtaining cross-sections of the cultures. Ultrathin sections (50–65 nm) were collected on copper grids and counterstained with uranyl acetate and Reynold's lead citrate. Sections were analyzed on an electron microscope (model 201; Philips Electronic Instruments Co., Mahwah, NJ). To confirm the extent of myelination of the control and treated cultures used for ultrastructural analysis, additional cultures treated identically and in parallel to those used for ultrastructural analysis were immunostained for MBP, and they were examined by immunofluorescence.

To quantify the number of Schwann cell/neurite units with complete or patchy basal lamina in control and TGF-β1–treated cultures, regions from at least two different coverslips for each condition were analyzed under the electron microscope.

Identification of TGF-β1 Receptors by Cross-linking

TGF-β1 was iodinated using the IODO-GEN iodination reagent (Pierce Chemical Co.) according to the manufacturer's instructions. Chemical cross-linking of iodinated TGF-β1 to cells was performed by a modification of described procedures (Wang et al., 1991). For these experiments collagen-coated 35-mm dishes were each plated with ~24 dissociated DRG's obtained from embryonic day 16 rat embryos. After cycling the cultures with antimitotic agents to remove nonneuronal cells, some of the cultures were seeded with ~1×10^6 Schwann cells per dish, and they were allowed to myelinate for 1 mo under standard conditions. The remaining neuron cultures were maintained in standard media. To perform the cross-linking reaction, cultures were washed four times with Dulbecco's PBS and incubated with binding buffer (Krebs-Ringer solution with 20 mM Hepes, pH 7.5, 0.5 mM $MgSO_4$, and 0.1% BSA) containing 25 pM ^{125}I-labeled TGF-β1 for 3 h at 4°C with gentle rotation. As a control for the specificity of the TGF-β1 binding, some cultures were incubated with competing amounts of unlabeled TGF-β1 (final concentration of 25 nM) in addition to the 25 pM ^{125}I-labeled TGF-β1. The cultures were then washed four times in binding buffer without BSA at 4°C and then incubated in this buffer containing *bis* (sulfosuccinimidyl) suberate (BS^3) (Pierce Chemical Co.) at a final concentration of 62 ng/ml for 15 min at 4°C with gentle shaking. The reaction was stopped by addition of Tris-HCl, pH 7.4, to a final concentration of 100 mM. The reaction buffer was removed, and the cultures were resuspended in SDS gel sample buffer. Samples were subjected to electrophoresis on a 7% SDS polyacrylamide gel and then developed with the PhosphorImager.

Statistical Analysis

Statistical analyses were performed using the Statview program (Abacus Concepts, Inc.). All data were analyzed statistically by analysis of variance followed by the Bonferroni/Dunn post-hoc test, except in the case of the TGF-β levels, which were subjected to the Fisher's protected least significant difference post-hoc test.

Results

Expression of TGF-β Isoforms by Schwann Cells and Neurons

As a first step in analyzing the potential role of TGF-β in the interactions of Schwann cells and neurons, we measured the expression of each of the three TGF-β isoforms by these cell types. To this end, we used a sensitive bioassay (Abe et al., 1994) to determine the levels of TGF-β in culture media con-

ditioned by Schwann cells and neurons grown alone or in coculture. In this assay, conditioned media from the cultures were incubated with a mink lung epithelial cell line stably transfected with an expression construct in which a TGF-β-inducible promoter (i.e., a portion of the PAI-1 promoter) was fused to a luciferase reporter gene. TGF-β stimulates these cells to synthesize luciferase in a dose-dependent manner. During incubation with the MLEC, we also added a series of commercially available neutralizing antibodies to the conditioned media. One antibody blocked the activity of all three TGF-β isoforms; the other antibodies were isoform specific. The decrease in the amount of luciferase synthesized in the presence of these antibodies represented TGF-β–specific activity.

Using this assay, we first determined the concentration of total (latent plus active) and active TGF-β in media conditioned by DRG neurons, Schwann cells, and neuron/Schwann cell cocultures under myelinating (serum plus ascorbic acid) and nonmyelinating (serum alone) conditions. Results of three separate experiments are summarized in Fig. 1. We found significant and comparable amounts of total TGF-β activity expressed by each cell type, with the most activity in the cultures of neurons and the least activity in the Schwann cell/neuron cocultures. Very little active TGF-β was detected in any of the cultures, with active levels consistently measured in the 4–5 pg/ml range compared to total levels in the 300–500 pg/ml range. Thus, in each case, the majority of the TGF-β activity present in the culture media is latent. Using isoform-specific antibodies, we also determined which of the three mammalian TGF-β isoforms are released by these cells. These studies demonstrated that all three TGF-β isoforms are released by neurons and Schwann cells, with the proportions varying by cell type. Neurons principally release TGF-β2 and -β3, whereas Schwann cells principally release TGF-β1 and -β2. The amount of the individual isoforms, when added together, exceeded the amount independently measured with an anti–TGF-β1,2,3 monoclonal antibody potentially reflecting less efficient inhibition of each isoform with this antibody. Of particular note, the levels of TGF-β1, as a proportion of the TGF-β activity, drop significantly in the neuron/Schwann cell cocultures in comparison to the levels of TGF-β1 in the Schwann cell cultures. By contrast, the levels of TGF-β2 are similar in each case, and the relative levels of TGF-β3 are elevated in the cocultures compared to Schwann cells alone. These results suggest that there is a differential regulation of TGF-β isoforms. TGF-β1 expression appears to be specifically downregulated as a result of coculture, suggesting it may have a role in mediating axon/Schwann cell interactions.

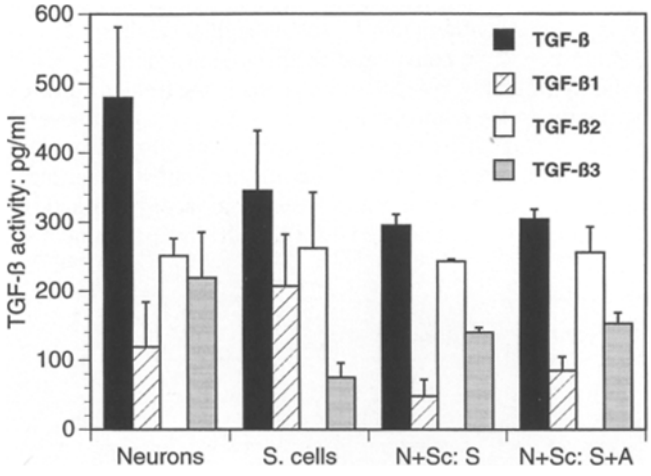

Figure 1. TGF-β levels in Schwann cells, neurons, and Schwann cell/neuron cocultures. Conditioned serum-free media were collected from cultures of neurons, Schwann cells, and neuron/Schwann cell cocultures that had previously been maintained in media containing serum (*N+Sc:S*) or serum plus ascorbic acid (*N+Sc:S+A*) to promote myelination. Total levels of TGF-β, TGF-β1, TGF-β2, and TGF-β3 were determined in three separate experiments; standard error bars are shown. Individual TGF-β isoform levels shown were corrected for cross-reactivity of the isoform-specific antibodies, as described in the Materials and Methods. The levels of TGF-β1, relative to the TGF-β activity, are significantly less in the neuron/Schwann cell cocultures compared to the Schwann cell cultures ($P < 0.005$), whereas the relative TGF-β3 activity is significantly greater ($P < 0.05$).

TGF-β1 Promotes NCAM and SCIP Expression and Antagonizes the Effects of Forskolin on Schwann Cells

To examine the potential significance of the regulated expression of TGF-β1 in these cultures, we first characterized its effects on the expression of Schwann cell markers. For these studies, we grew Schwann cells in media, with or without serum, containing 1 or 10 ng/ml of TGF-β1, 10 μM forskolin, 10 μM forskolin with 10 ng/ml of TGF-β1, or without supplements (control). As previously noted (Rogister et al., 1993), TGF-β dramatically alters the morphology of Schwann cells. Thus, cells treated with TGF-β, with forskolin, or with both

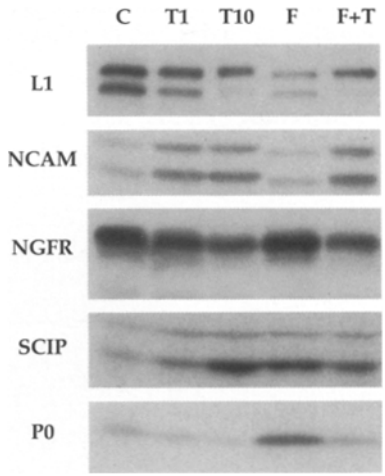

Figure 2. Effect of TGF-β1 on Schwann cell markers. Schwann cells were grown in serum containing media supplemented with either 1 ng/ml of TGF-β1 (*T1*), 10 ng/ml of TGF-β1 (*T10*), 10 μM forskolin (*F*), or 10 ng/ml of TGF-β1 plus 10 μM forskolin (*F+T*). Lysates were prepared, fractionated by SDS-PAGE, and blotted. The nitrocellulose blot was cut into strips that were probed with antibodies to L1 (which recognized bands of 220 and 200 kD), NCAM (140 and 120 kD), the NGF receptor (75 kD), SCIP (40 kD), and P0 (25 kD). After incubation with the primary antibodies, each strip was incubated with ^{125}I protein A and exposed for autoradiography.

agents were more polygonal and less spindle shaped in appearance than control cells and appeared to be more frequently in contact with one another (data not shown). In addition, although not specifically investigated, Schwann cell survival after 1 wk appeared to be better in the defined media supplemented with TGF-β1 or forskolin than in the defined media alone. At 1 wk, Schwann cell lysates were prepared and analyzed by Western blotting to quantitate the effect of TGF-β and forskolin on the expression of L1, NCAM, the p75 NGF receptor, the transcription factor SCIP, and the myelin protein P0. An example of a typical Western blot is shown in Fig. 2, and results from several such experiments are summarized in Table I.

In general, addition of TGF-β1 appears to promote a pre- or nonmyelinating Schwann cell phenotype. Thus, treatment with high or low concentrations of TGF-β1 resulted in increased expression of NCAM, which is present on nonmyelinating Schwann cells. TGF-β1 also generally suppressed the basal expression of the myelin protein P0 and inhibited its induction by forskolin. TGF-β1 also reduced expression of the NGF receptor and of L1 (particularly in serum-containing media). The magnitude of the effects of both forskolin and TGF-β1 on Schwann cells was generally not as pronounced in the presence of serum, consistent with other studies (Morgan et al., 1994). Of particular note, we observed a consistent and substantial induction of SCIP expression by TGF-β1, both in the presence and absence of serum (see Fig. 2). The basal level of SCIP expression in Schwann cells grown in media without serum appeared to be lower than in cells grown with serum which may account, in part, for the generally greater induction of SCIP expression observed with forskolin under defined media conditions (data not shown).

In other studies, we found that TGF-β1 had no effect on the expression of NCAM and L1 by neurons that were similarly treated (data not shown), indicating that these effects were specific to Schwann cells in this system.

TGF-β Inhibits Schwann Cell Myelination

The effect of TGF-β1 on Schwann cell markers described above suggested that TGF-β1 might antagonize the differentiation of Schwann cells toward the myelinating phenotype. To investigate this possibility directly, we added TGF-β1 to cocultures of neurons and Schwann cells, and we characterized the extent of myelination after 1 wk. Results of a typical experiment are shown in Fig. 3, and they are summarized in Table II. While untreated cocultures extensively myelinated (Fig. 3 *A*), addition of 10 ng/ml of TGF-β1 completely inhibited myelination (Fig. 3 *E*). Although no myelin sheaths were present in the treated cultures, we often observed a small amount of particulate staining for MBP in the Schwann cells, suggesting there may be some residual synthesis. In addition, as will be considered further below, there appeared to be significantly fewer Schwann cells in the treated cultures compared to the controls (Fig. 3, *F* vs *B*). Even after several weeks of TGF-β1 treatment, no myelin was observed in any of the cultures treated with 10 ng/ml (data not shown), indicating that there was a complete inhibition of myelination. This effect was not limited to TGF-β1, since treatment of

Table II. TGF-β1 Inhibits Schwann Cell Myelination

Day of TGF-β1 addition	Concentration of added TGF-β1	Number of myelin segments per coverslip	Inhibition (%)
	ng/ml		
0	0	2,943 ± 567	–
0	1	671 ± 61	77.2
0	10	0 ± 0	100
2	10	5 ± 0.5	99.8

The number of myelin segments was determined in Schwann cell/DRG cocultures grown in the absence or presence of 1 or 10 ng/ml TGF-β1. The TGF-β1 was either added at the time the cultures were switched to standard media containing ascorbic acid (day 0) or after the cultures had been maintained in this media for 2 d (day 2). After a total of 8 d of growth under myelin-promoting conditions, the cultures were fixed and the number of MBP-positive segments was determined. The mean values and SEM presented are from the myelin counts of four day 0 cultures at each concentration and two day 2 cultures from a representative experiment. The number of myelin segments in the day 0 control cultures was significantly different from that of the cultures treated with TGF-β1 on day 0 and day 2 ($P < 0.001$).

Table I. Effect of TGF-β1 and Forskolin on the Expression of Schwann Cell Markers

A Defined media

	L1	NCAM	NGFR	SCIP	P0
T1	1.48 ± 0.10	3.45 ± 0.85	0.76 ± 0.06	1.77 ± 0.42	2.70 ± 0.95
T10	0.87 ± 0.03	6.37 ± 2.31	0.50 ± 0.06	3.10 ± 0.66	0.73 ± 0.23
F	0.53 ± 0.03	2.85 ± 1.05	0.77 ± 0.18	35.03 ± 12.88	8.20 ± 1.19
F+T	0.70 ± 0.12	4.90 ± 1.73	0.73 ± 0.12	13.30 ± 4.40	5.33 ± 1.96

B Serum-containing media

	L1	NCAM	NGFR	SCIP	P0
T1	0.75 ± 0.05	1.56 ± 0.09	0.84 ± 0.04	2.19 ± 0.46	0.62 ± 0.03
T10	0.44 ± 0.08	1.63 ± 0.15	0.54 ± 0.04	8.74 ± 5.06	0.53 ± 0.11
F	0.30 ± 0.01	0.98 ± 0.18	1.03 ± 0.01	8.38 ± 5.27	2.36 ± 0.06
F+T	0.43 ± 0.04	2.07 ± 0.47	0.56 ± 0.02	3.40 ± 1.45	1.01 ± 0.10

Primary Schwann cells were grown in defined media (*A*) or media containing 10% fetal calf serum (*B*) with TGF-β1 at 1 ng/ml (*T1*) or 10 ng/ml (*T10*), 10 μM forskolin (*F*), or forskolin plus 10 ng/ml of TGF-β1 (*F+T*). The expression of L1, NCAM, the p75 NGF receptor, P0, and SCIP were determined by Western blotting, and they are given relative to levels in untreated (control) Schwann cells grown in the same media. Mean values and SEM from three to six determinations are presented in *A*, and mean values and range from duplicate determinations are presented in *B*.

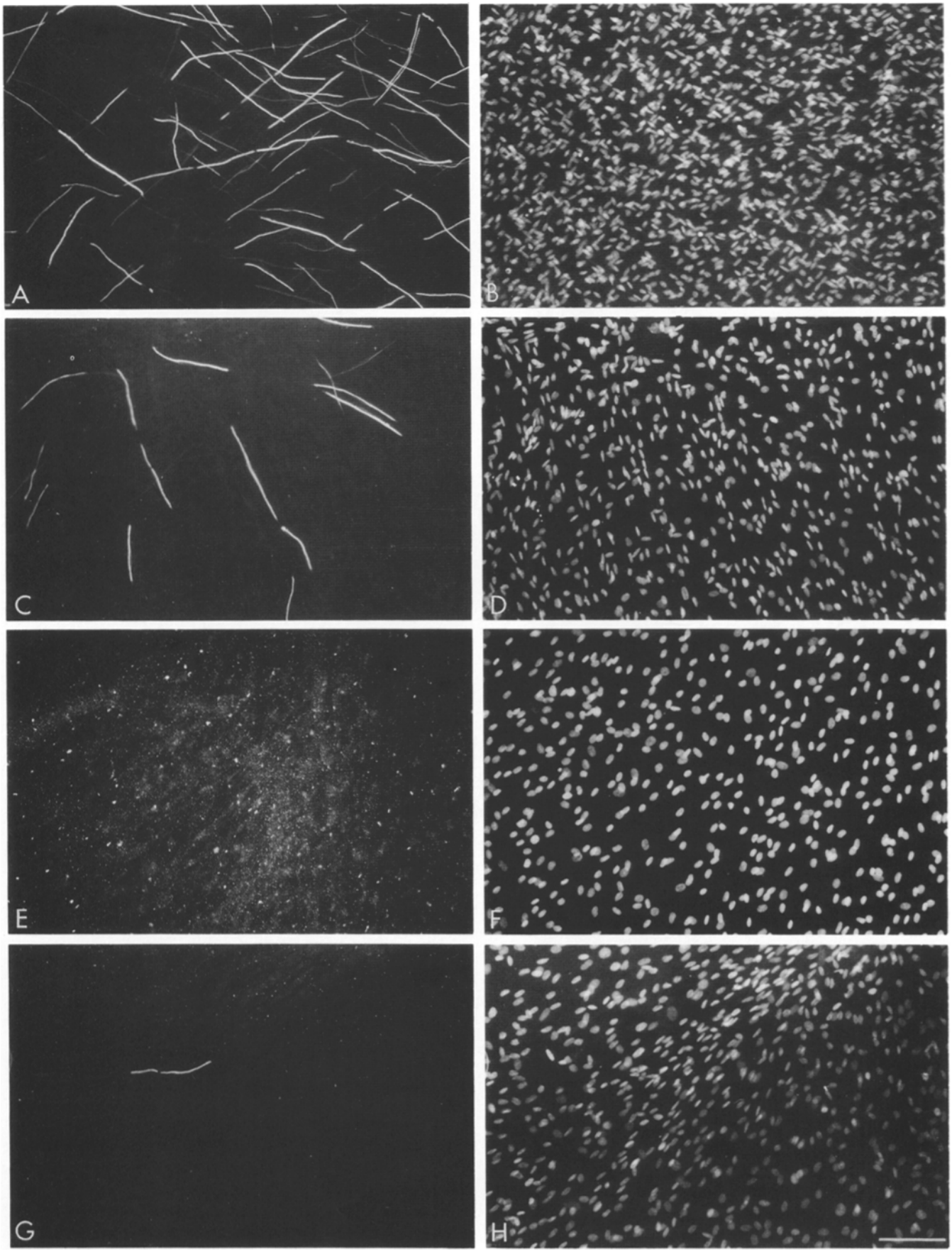

Figure 3. TGF-β1 inhibits myelination. A control culture (*A* and *B*) and cultures treated with 1 ng/ml of TGF-β1 (*C* and *D*) or with 10 ng/ml of TGF-β1 (*E* and *F*) immediately after adding media that promotes myelination and a culture treated with 10 ng/ml of TGF-β1 commencing 48 h after adding such media (*G* and *H*) are shown. Panels on the left side of the figure (*A*, *C*, *E*, and *G*) are immunofluorescent micrographs of the cultures stained for myelin basic protein; the identical fields demonstrating Schwann cell nuclei stained with a nuclear dye are shown on the right (*B*, *D*, *F*, and *H*). Note that there are significantly fewer Schwann cells in the TGF-β1–treated cultures. Bar, 100 μm.

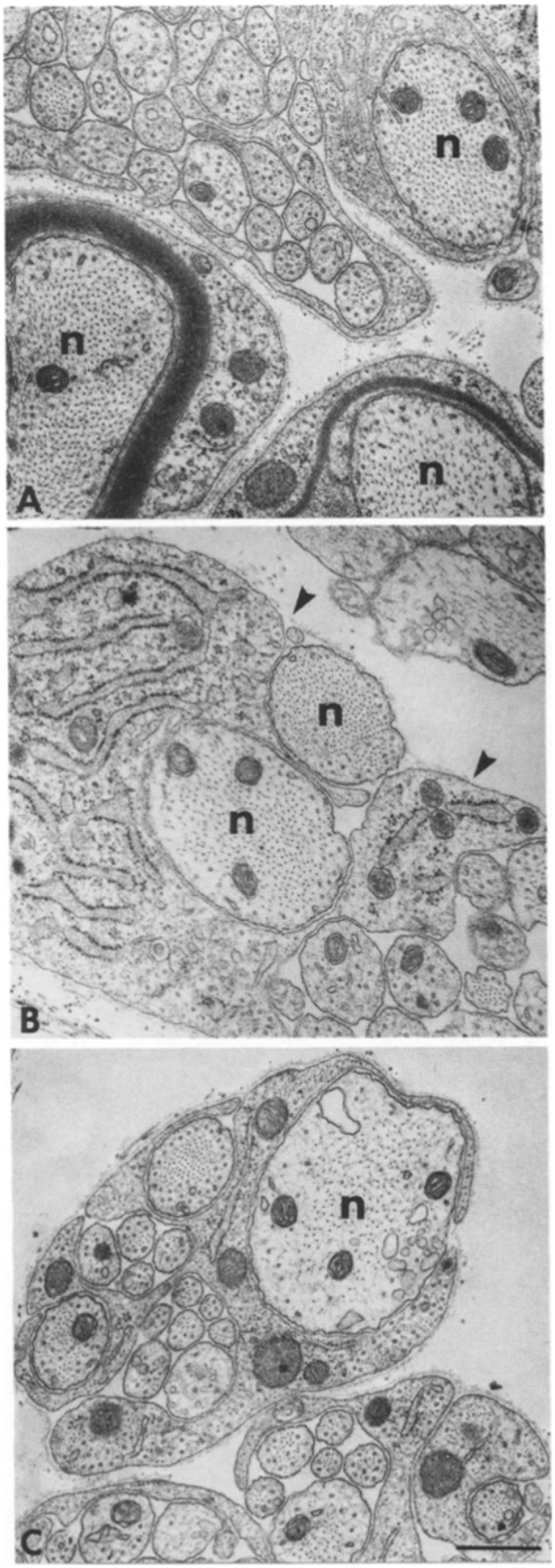

Figure 4. Ultrastructure of control and TGF-β1–treated cultures. Electron micrographs of control (*A*) and TGF-β–treated cultures (*B* and *C*). The culture shown in *B* was treated with 10 ng/ml of TGF-β1 from the time the culture was switched to media promoting myelination; TGF-β was added to the culture shown in *C* 2 d after switching to this media. Large neurites (*n*) that are myelinated or loosely wrapped are visible in the control culture; similar sized neurites are only partially segregated off in *B*, or they are ensheathed together with small nerve fibers in *C*. An area of patchy basal lamina present in the TGF-β–treated culture in *B* is indicated by the arrowheads. Bar, 0.5 μm.

cocultures with 10 ng/ml of TGF-β2 also resulted in a complete inhibition of myelination in the cocultures (data not shown). Addition of 1 ng/ml TGF-β1 to the cocultures significantly inhibited myelin formation as well, although the inhibition was incomplete (Fig. 3 *C*). In three separate experiments, the average number of myelin segments in cultures treated with 1 ng/ml was reduced from 66% to >90%. After several weeks, cultures treated with 1 ng/ml of TGF-β1 did eventually myelinate quite extensively suggesting that, at this concentration, the principal effect of TGF-β1 is to delay the process of myelination.

To determine whether there was a critical window for the inhibitory effects of TGF-β1 on myelination, we added TGF-β1 at varying times of Schwann cell/neuron coculture. In one set of studies, we pretreated the Schwann cells with 10 ng/ml of TGF-β1 for 1 wk before adding them to cultures of neurons. Alternatively, we pretreated the neurons for 1 wk, removed the TGF-β1, and then inhibited any residual TGF-β1 with a pan–TGF-β blocking antibody or by brief trypsinization before adding Schwann cells. In both cases, cocultures subsequently myelinated comparably to control cultures (data not shown). These results indicate that TGF-β1 does not irreversibly block the ability of Schwann cells to form myelin. We next added TGF-β1 at various times after establishment of the cocultures. We previously noted that 3 d after switching cocultures to medium supplemented with serum and ascorbate, the first myelin sheaths are detectable by immunofluorescence; many of the remaining Schwann cells appear to be in an ensheathing or premyelinating relationship with axons (Einheber et al., 1993). Strikingly, addition of 10 ng/ml of TGF-β1 just before the onset of myelination, i.e., 48 h after switching to media supplemented with serum and ascorbate, also effectively blocked myelination in the cocultures (see Fig. 3 *G* and Table II). In preliminary experiments, addition of TGF-β1 as late as 5 d after coculture appeared to profoundly inhibit myelin formation (data not shown), suggesting that Schwann cells in an early stage of myelination are still efficiently inhibited by TGF-β1. By contrast, once extensive amounts of myelin have formed (i.e., after 1 wk of coculture), treatment with TGF-β1 for an additional week or longer did not result in appreciable degeneration of myelin sheaths that had already formed (data not shown). Taken together, these results indicate that TGF-β1 must be present during the period of active myelination to inhibit this process effectively; pretreatment does not irreversibly inhibit Schwann cells from forming myelin, nor does short term treatment cause breakdown of mature myelin sheaths that have already formed.

TGF-β Results in Defects of Schwann Cell Ensheathment and Basal Lamina Formation

To determine more precisely the effect of TGF-β1 on Schwann cell ensheathment and myelination, we performed an ultrastructural analysis of cocultures of Schwann cells and

Table III. TGF-β1 Inhibits Schwann Cell Basal Lamina Formation

Day of TGF-β1 addition	Concentration of added TGF-β1	Number of Schwann cells examined	Percent of Schwann cells with a basal lamina		
			Complete	Partial	Absent
	ng/ml				
0	0	229	68.6	31.4	0
0	10	165	15.2	80.6	4.2
2	10	205	59.5	40.5	0

The extent of basal lamina formation in Schwann cell/DRG cocultures grown in the absence or presence of 10 ng/ml TGF-β1 was determined from electron micrographs. The TGF-β1 was added either at the time the cultures were switched to standard media containing ascorbic acid (day 0) or after the cultures had been maintained in this media for 2 d (day 2). After 8 d of growth under myelinating conditions, the cultures were fixed and processed for electron microscopy. The Schwann cell basal lamina was scored as complete if it formed a continuous, uninterrupted layer around Schwann cells associated with neurites. The percentage of Schwann cells with complete basal lamina in the control cultures and cultures treated with 10 ng/ml of TGF-β1 on day 2 was greater than in the cultures treated with TGF-β1 on day 0 ($P < 0.0001$).

neurons maintained with or without 10 ng/ml of TGF-β1 for 8 d (see Fig. 4). Cultures treated with 10 ng/ml TGF-β1 demonstrated a variety of abnormalities when compared to control cultures, including defects of ensheathment, complete failure to form myelin, and partial defects of basal lamina formation (Fig. 4 *B*). In the treated cultures, Schwann cells typically extended relatively short processes into the nerve fiber bundles and only partially separated nerve fibers. By light microscopy, this abnormality of ensheathment appeared to be more prominent in the center of the coverslip, where bundles of unensheathed fibers were frequently observed, than in peripheral regions where there were more Schwann cells (data not shown). However, ensheathment was clearly abnormal and more rudimentary in the periphery compared to control cultures. In addition, the rough ER was quite prominent in many of the treated Schwann cells which frequently contained cisternae of swollen ER decorated with ribosomes (see Fig. 4 *B*). Finally, the amount of basal lamina formed by TGF-β–treated Schwann cells appeared to be thinner and less complete than in control cultures. Schwann cells containing only a partial basal lamina were much more common in the treated cultures than in the control cultures, and cells devoid of any basal lamina were present in the treated cultures, but not in the control sections that were analyzed. These effects of TGF-β1 on basal lamina formation are quantitated in Table III.

We also examined the morphology of cocultures treated with 1 ng/ml of TGF-β1, as well as cocultures placed on 10 ng/ml after 2 d of culture in the presence of serum and ascorbate. In general, cultures treated with 1 ng/ml of TGF-β1 demonstrated defects similar to those described above, although the abnormalities were not as pronounced. Ensheathment of nerve fibers had progressed further, occasional myelinated fibers were encountered, and defects of the basal lamina were not as severe (data not shown). Cultures that were treated with 10 ng/ml of TGF-β1 after a 2-d delay, although appearing to ensheathe more normally, still contained very few myelinated fibers. This failure to myelinate may reflect the fact that fewer large diameter neurites appeared to be segregated off in a 1:1 relationship with Schwann cells in comparison to control cultures (see Fig. 4 *C*). Of particular note, the Schwann cell basal lamina in these cultures was comparable to controls (see Table III). Therefore, these results suggest that the ability of TGF-β1 to inhibit myelination in these cultures is likely to be independent of its effects on basal lamina formation.

To analyze further the basal lamina defect in cultures treated with TGF-β1, we stained control and treated cultures with a polyclonal anti-laminin antibody 1 wk after adding ascorbic acid (shown in Fig. 5). In the control cultures, Schwann cells were actively myelinating and expressed readily detectable levels of MAG (Fig. 5 *C*). Expression of laminin by these early myelin segments was quite robust, and localized at the outer surface of myelin sheaths, as well as that of Schwann cells that had just begun to myelinate (Fig. 5 *E*); nonmyelinating Schwann cells were also brightly stained, demonstrating a more fibrillar pattern of expression over their surface. By contrast, the pattern of laminin staining in the treated cultures, while generally similar, was notably attenuated and less distinct (Fig. 5 *F*). Schwann cells also appeared to be larger and more flattened in the treated cultures (Fig. 5 *B*), and they did not stain with MAG antibodies (Fig. 5 *D*). These results suggested that TGF-β inhibited laminin expression. To quantitate this effect, we analyzed the expression of laminin in treated and control cocultures by Western blot analysis. As shown in Fig. 6, the total amount of laminin present in the treated cocultures was significantly reduced. Quantitative analysis indicated that treated cultures contained ~50% of the amount of laminin present in control cultures; in contrast, tubulin expression in these cultures was comparable (data not shown). It is of interest that the laminin antibody only recognized one or both of the laminin β chains in the treated and control cultures, but not the α chain, indicating that the major laminin component in these cultures is not laminin-1, but rather, a laminin isoform. In preliminary studies, we have detected both α2 (merosin) and β2 (S-laminin) chains in these cultures, with α2 levels in particular showing a significant decrease in the TGF-β-treated cultures (data not shown).

TGF-β1 is a Mitogen for Purified Schwann Cells, but it Inhibits the Proliferation of Schwann Cells in Coculture with Neurons

In view of previous reports that TGF-β is a Schwann cell mitogen (Eccleston et al., 1989; Ridley et al., 1989), we were surprised to note that there were consistently fewer Schwann cells in the cocultures treated with TGF-β1 than in the control cultures (see Fig. 3). These results suggested that TGF-β might have a paradoxical inhibitory effect on the proliferation of Schwann cells induced by neurons. To test this possibility, we compared the effect of TGF-β on the proliferation of purified Schwann cells to the effect on Schwann cells cocultured with neurons. Consistent with earlier reports, we found that TGF-β1 is a mitogen for purified Schwann cells, resulting in a nearly seven-fold increase in their labeling index (see Table IV). By contrast, TGF-β1 is a strong antagonist of the proliferation induced by contact with neurites, reducing the labeling index by ~60% at 1 ng/ml and by ~75% at 10 ng/ml; a representative experiment is shown in Fig. 7, and several such experiments are summarized in Table V. This inhibition of proliferation in the co-

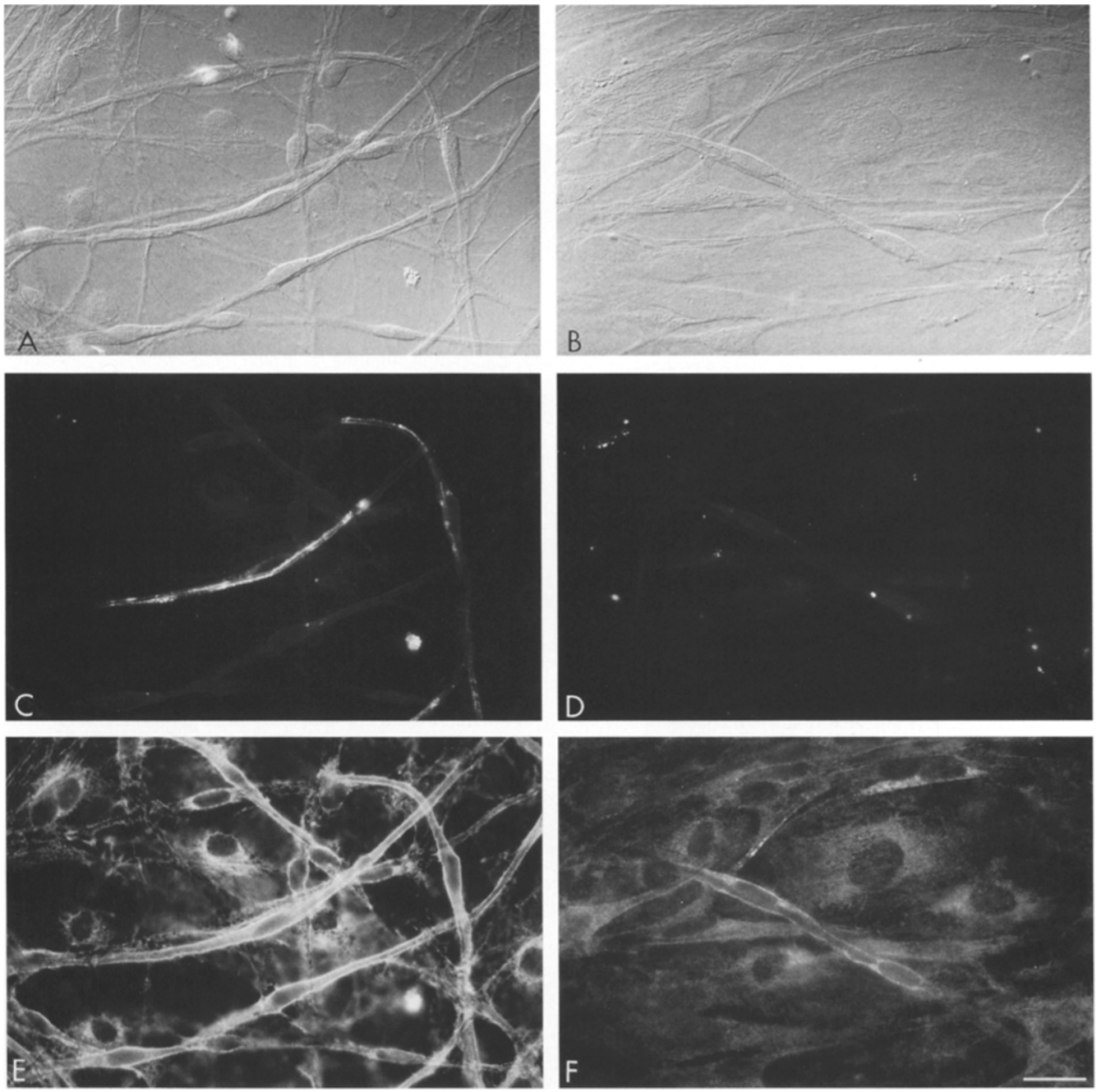

Figure 5. Laminin staining of TGF-β1-treated cultures. Control cultures (*A*, *C*, and *E*) or cultures continuously treated with TGF-β1 at 10 ng/ml (*B*, *D*, and *F*) were fixed after 1 wk and visualized by Nomarski optics (*A* and *B*), or they were immunostained for MAG (*C* and *D*) or laminin (*E* and *F*). Bar, 25 μm.

cultures was observed when TGF-β1 was added in media containing serum plus ascorbic acid (SA media) either immediately upon switching from serum-free media to SA media (condition A) or 2 d after such a switch (condition B). We also maintained the cocultures in media with serum but not ascorbic acid for 1 wk to allow Schwann cells to repopulate neurites partially while preventing basal lamina formation or myelination (Fernandez-Vallé et al., 1993); we then added the TGF-β1 in SA media (condition C). Under all three conditions, TGF-β1 significantly inhibited proliferation and any subsequent myelin formation. In addition, proliferation of Schwann cells was also reduced in cocultures maintained in serum-free defined media in the presence of TGF-β1 (data not shown). Thus the effects of TGF-β1 on Schwann cell proliferation strikingly differ, depending on whether or not Schwann cells are associated with nerve fibers.

This effect of TGF-β1 on Schwann cell proliferation raised the possibility that the cultures treated with TGF-β1 failed to myelinate because of insufficient numbers of Schwann cells. In an effort to distinguish between the effect of TGF-β1 on

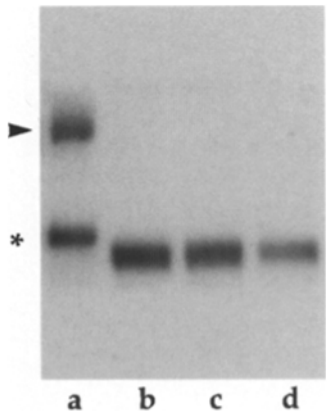

Figure 6. TGF-β1 inhibits laminin expression. Lysates from control neuron/Schwann cell cultures (*b*) or cocultures treated with 1 ng/ml of TGF-β1 (*c*) or 10 ng/ ml of TGF-β1 for 1 wk (*d*) were prepared, fractionated, and probed with an antilaminin polyclonal antibody, incubated with ^{125}I protein A, and visualized by autoradiography. In lane (*a*), 0.4 μg of laminin is shown for comparison. Note that the upper band (*arrowhead*) corresponding to the laminin α chain, which is present in the laminin control, is not expressed by the neuron/Schwann cell cultures. Also, the β chains (*asterisk*) are of lower relative molecular mass in the cocultures.

Schwann cell proliferation and differentiation, we plated Schwann cells onto cultures that contained a third fewer neurons than our typical paradigm before adding TGF-β1. Under these conditions, the ratio of Schwann cells to neurons was extremely high, and the number of Schwann cells in the control and treated cultures were comparable when cells were visualized with a dye that stains cell nuclei (data not shown). Nevertheless, no myelin was observed in any of the cultures, suggesting the effects of TGF-β1 on myelination are independent of its effects on proliferation.

Expression of TGF-β Receptors in Cocultures of Schwann Cells and Neurons

To identify whether neurons, Schwann cells, or both were targets of these effects of TGF-β, we determined the pattern of TGF-β receptor expression by cross-linking with iodinated TGF-β1. Cultures of neurons alone or myelinating cocultures were incubated with radioactive TGF-β1 and then briefly treated with the homobifunctional crosslinker BS^3. Lysates were then prepared, fractionated by SDS-PAGE, and exposed with a PhosphorImager. Results are shown in Fig. 8. In the Schwann cell/neuron cocultures, bands corresponding in size to ~70, 85, and a broad band from 200 to 250 kD were strongly labeled and readily apparent (Fig. 8, lane *c*). These bands correspond in size to TGF-β type I, type II, and type III receptors, respectively, that were previously defined in other systems by similar techniques (Wang et al., 1991). Specificity of this cross-linking pattern was indicated by the ability of excess, unlabeled TGF-β1 to block essentially all labeling (Fig. 8, lane *d*). By contrast to the strong expression of receptors in these cultures, very little labeling of receptors was observed in the cultures of neurons alone. As most of the neurons are likely to be either ensheathed or myelinated by Schwann cells in these mature cocultures, and in view of the minimal expression of receptors in the neuron cultures, these results suggest that Schwann cells are the principal cell type expressing TGF-β receptors in the cocultures. This result is consistent with the effect of TGF-β1 on Schwann cell markers (Table I) in contrast to its limited effect, if any, on neurons.

Table IV. TGF-β1 Stimulates Schwann Cell Proliferation in the Absence of Neurons

Concentration of added TGF-β1	Percent of BrDU-positive cells
ng/ml	
0	0.9 ± 0.4
1	6.3 ± 1.4
10	5.9 ± 1.2

Proliferation assays were performed on cultures of Schwann cells maintained for 72 h in standard media with or without TGF-β1. The percent of BrDU-positive cells after a 3-h pulse was determined from three or four coverslips per condition from a representative experiment; mean values and SEM are shown. The percent of BrDU-positive cells in the control cultures was significantly different from that of the cultures treated with 1 ng/ml and 10 ng/ml TGF-β1 ($P < 0.05$).

Table V. TGF-β1 Inhibits Schwann Cell Proliferation Induced by Coculture with Neurons

Condition	Concentration of added TGF-β1	Percent of BrDU-positive cells
	ng/ml	
A	0	22.4 ± 0.3
	1	7.7 ± 1.0
	10	6.4 ± 0.6
B	0	13.5 ± 3.2
	1	6.1 ± 1.5
	10	3.0 ± 0.7
C	0	9.0 ± 0.5
	10	1.5 ± 0.3

The effect of TGF-β1 on the proliferation of Schwann cells in coculture with neurons was determined under three different culture conditions. In condition A, neurons seeded with Schwann cells were maintained in a defined media for 3 d, and then switched to standard media containing ascorbic acid with or without TGF-β1 for 20 h. In condition B, Schwann cell/neuron cocultures were grown in a defined media for 3 d and standard media containing ascorbic acid for 2 d before addition of the TGF-β1. In condition C, the cocultures were maintained in standard media for 7 d, and then switched to standard media containing ascorbic acid with or without TGF-β1. The percent of BrDU-positive cells after a 3-h labeling period was determined from duplicate or triplicate coverslips from representative experiments for conditions A and B and from six coverslips for condition C; mean values and SEM are presented. In all three conditions, the percent of BrDU-positive cells in the control cultures were significantly different than those of cultures that were treated with 10 ng/ml TGF-β1 ($P < 0.05$).

Discussion

In this paper, we have investigated the expression of TGF-β by neurons and Schwann cells, and we have characterized the effect of TGF-β1, an abundant isoform produced by Schwann cells, on their proliferation and differentiation. We have found that TGF-β1, which induces the proliferation of Schwann cells grown alone, dramatically inhibits the proliferation, myelination, and basal lamina formation of Schwann cells in coculture with neurons. These results suggest that TGF-β1 may be an important mediator of axon/Schwann cell interactions, regulating Schwann cell proliferation and differentiation during development and, as will be discussed, after nerve injury.

Expression of TGF-β Isoforms by Schwann Cells and Neurons

We have obtained compelling evidence that both Schwann

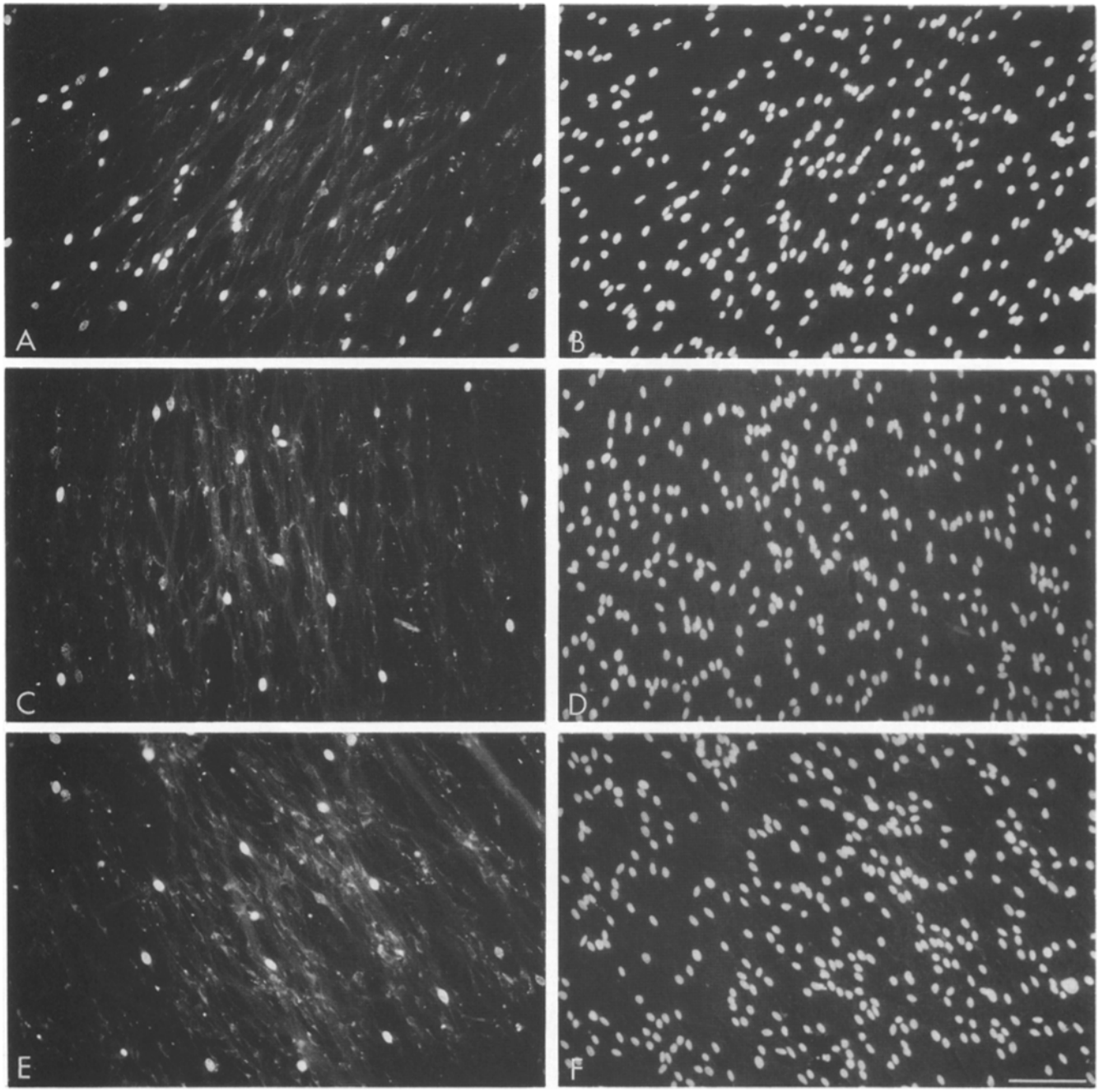

Figure 7. TGF-β1 inhibits Schwann cells proliferating on neurites. Schwann cells were seeded onto cultures of sensory neurons, maintained in defined media, and then switched to SA media without TGF-β1 (*A* and *B*) or with 1 ng/ml of TGF-β1 (*C* and *D*) or 10 ng/ml of TGF-β1 (*E* and *F*). After 24 h, BrDU was added for 3 h and cultures were fixed and stained with an anti-BrDU antibody (*A*, *C* and *E*), or the same fields were visualized with a nuclear dye (*B*, *D*, and *F*). Bar, 100 μm.

cells and sensory neurons synthesize all three TGF-β isoforms, although the proportions vary by cell type. Thus, Schwann cells secrete a higher proportion of TGF-β1 and -β2 compared to neurons that express relatively more TGF-β2 and -β3. This work significantly extends previous investigations on the expression of TGF-β isoforms by neurons and Schwann cells in the peripheral nerve (Flanders et al., 1991; Unsicker et al., 1991; Scherer et al., 1993) and in vitro (Mews and Meyer, 1993; Rogister et al., 1993). By using a bioassay, we also determined the amount of total vs active TGF-β for each isoform. In each case, the majority of the TGF-β detected was latent, with active levels in the 3–5 pg/ml range. This concentration of TGF-β has significant biological effects in many systems (reviewed briefly in Abe et al., 1994). Whether these levels are biologically significant for Schwann cells is not yet clear. It should be noted that we have measured the amount of TGF-β present in the culture media, and we do not yet know whether this accurately reflects the levels of TGF-β associated with the cells and their extracellular matrix. Moreover, the concen-

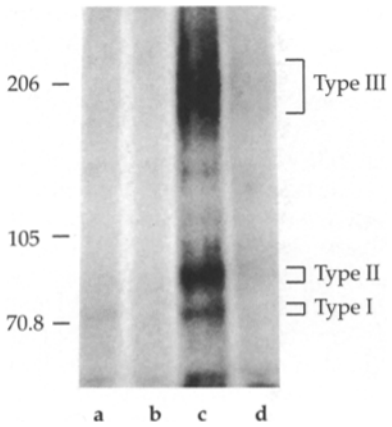

Figure 8. Expression of TGF-β1 receptors in cocultures. Iodinated TGF-β1 was cross-linked to cultures of neurons alone (lanes *a* and *b*) or heavily myelinated neuron/Schwann cell cocultures (lanes *c* and *d*) in the absence (lanes *a* and *c*) or presence (lanes *b* and *d*) of an excess of unlabeled TGF-β1. Cell lysates were prepared, fractionated by SDS-PAGE, and they were exposed with a PhosphorImager. The major bands that are visible in the myelinating cocultures correspond in size to the three major classes of TGF-β receptors as indicated to the right; molecular weight markers are provided on the left.

tration of TGF-β present in the periaxonal space could be higher than the amounts detected in the media, particularly if TGF-β is released into this site by neurons or Schwann cells, as may be the case for other growth factors, notably acidic FGF that has been localized immediately subjacent to the axolemma (Elde et al., 1991). Immunolocalization of TGF-β and its receptors should clarify this issue.

It is of particular interest that there is a specific decrease in the amount of TGF-β1 in the conditioned media from the cocultures, particularly in comparison to the Schwann cell cultures. By contrast, TGF-β2 levels in the cocultures remain essentially unchanged and TGF-β3 levels may even increase in comparison to the Schwann cell cultures. This reduction in the amount of TGF-β1 occurs despite the increased number of cells present in the cocultures (i.e., both neurons and Schwann cells). Also, this decrease was observed in cocultures maintained without ascorbic acid, which therefore lacked a basal lamina to which TGF-β1 might bind. These results indicate that expression of TGF-β1 is reduced as a result of neuron/Schwann cell interactions. Strong support that TGF-β1 levels are indeed regulated by Schwann cell/neuron interactions was provided by a recent report that TGF-β1 mRNA levels increase dramatically after sciatic nerve crush, and that they fall again as nerve fibers regenerate and contact the Schwann cells again (Scherer et al., 1993); interestingly, a converse pattern of expression was noted for TGF-β3. Similarly, TGF-β1 protein levels were noted to increase substantially at the site of sciatic nerve crush by immunocytochemistry (Rogister et al., 1993). Finally, treatment of Schwann cells with forskolin, which mimics many of the effects of axonal contact, downregulates the expression of TGF-β1 (Mews and Meyer, 1993; Scherer et al., 1993). Taken together, these findings indicate that expression of TGF-β1 by the Schwann cell is significantly and specifically downregulated by axons, and that it increases substantially after peripheral nerve injury. As will be discussed further, these findings and the effects of TGF-β1 on Schwann cells support a role for TGF-β1 during Wallerian degeneration.

TGF-β Has Dual Effects on Schwann Cell Proliferation That Depend on Neuronal Contact

TGF-β1 and -β2 were previously reported to be Schwann cell mitogens (Eccleston et al., 1989; Ridley et al., 1989; Davis and Stroobant, 1990; Schubert, 1992; Rogister et al., 1993) in contrast to their inhibition of cell proliferation in many other cell types (Massagué, 1990; Sporn and Roberts, 1992). We have confirmed these results, observing an approximate seven-fold increase in the labeling index of Schwann cells treated with TGF-β1. It is not yet known whether TGF-β acts directly as a mitogen or, alternatively, promotes Schwann cell proliferation by inducing expression of another growth factor, as has been reported in the case of its mitogenic effect on aortic endothelial cells (Leof et al., 1986). In striking contrast to its mitogenic effect on purified Schwann cells, we found that TGF-β1 is a potent inhibitor of the Schwann cell proliferation engendered by neurons. Because Schwann cells are normally quiescent unless they are in contact with axons (Wood and Bunge, 1975; Salzer et al., 1980), this TGF-β effect could reflect a direct inhibition of Schwann cell proliferation or, an indirect effect, i.e., interfering with the ability of the axon to deliver its mitogenic signal. The rapid inhibition of proliferation in the Schwann cell/neuron cocultures and the apparent lack of significant numbers of TGF-β receptors on neurons, however, suggest that this is a direct effect on Schwann cell proliferation and is not caused by inhibition of the expression of the neuronal mitogen. These results also emphasize that the response of Schwann cells to polypeptide growth factors can dramatically differ depending on their association with nerve fibers.

TGF-β Regulated Schwann Cell Differentiation

A major finding of this study is that TGF-β appears to drive Schwann cells towards a non- or premyelinating phenotype, inhibiting the expression of the p75 NGF receptor and partially blocking the forskolin mediated induction of P0. These findings confirm recent reports that TGF-β1 inhibits p75 NGF receptor mRNA (Mews and Meyer, 1993; Rogister et al., 1993), as well as the forskolin induction of P0 protein and mRNA (Mews and Meyer, 1993; Morgan et al., 1994). The effects of TGF-β1 on cell adhesion molecule expression by Schwann cells are more complex. We found that TGF-β treatment of Schwann cells results in a consistent increase in NCAM expression that ranged from 1.6-fold in the presence of serum to 6-fold in serum-free media (Table I). By contrast, TGF-β1, particularly at high concentrations in the presence of serum, significantly suppressed L1 expression whereas at low concentrations in defined media, TGF-β1 had a minimal or even a stimulatory effect on L1 expression. These latter results may be compared to previous reports that TGF-β1 increases the expression of NCAM protein and mRNA by two- to three-fold in the 3T3 fibroblast line (Roubin et al., 1990), and that it upregulates L1, but not NCAM, protein and mRNA in a population of postnatal murine cerebellar glial cells (Saad et al., 1991). Taken together,

these findings indicate that TGF-β1 has complex effects on adhesion molecule expression that vary by cell type, and they demonstrate that expression of NCAM and L1 can be independently regulated in Schwann cells. Although we have not specifically investigated the functional significance of the changes in cell adhesion molecule expression induced by TGF-β, we have noted that treated Schwann cells tend to aggregate more readily after trypsinization than control cells (Einheber, S., and J. Salzer, unpublished observations), suggesting that TGF-β1 promotes adhesive interactions.

We have also characterized the effects of TGF-β1 on the expression of the Schwann cell transcription factor SCIP. TGF-β1 increased SCIP expression from two- to eight-fold in the presence of 1 or 10 ng/ml of TGF-β1, respectively. Since SCIP expression has been correlated with the premyelinating phenotype (Monuki et al., 1990), these results provide additional evidence that TGF-β1 promotes the transition of Schwann cells to an early, nonmyelinating phenotype. TGF-β1, together with agents that elevate intracellular cAMP, represent independent mechanisms for elevating SCIP expression in Schwann cells. However, although both TGF-β1 and forskolin induce SCIP expression, they have contrasting effects on the Schwann cell phenotype, with TGF-β1 suppressing and forskolin promoting the myelinating phenotype. Because SCIP has been demonstrated to repress transcription of the P0 and NGF receptor promoters (Monuki et al., 1990; He et al., 1991), these findings raise the possibility that the inhibition of P0 and NGF receptor expression by TGF-β1 is related to this increased SCIP expression. (The effect of TGF-β1 on SCIP does not explain its ability to antagonize the forskolin induction of P0 expression, since SCIP levels in Schwann cells treated with both forskolin and TGF-β1 were lower on average than the levels in cells treated with forskolin alone.) The relationship of this increase in SCIP expression, if any, to the observed changes in NCAM and L1 levels, remains to be determined.

TGF-β1 Inhibits Schwann Cell Myelination and Basal Lamina Formation

One of the most dramatic findings of this paper, the essentially complete inhibition of myelination in the cocultures treated with TGF-β1 and -β2, also supports a role for TGF-β in promoting a pre- or nonmyelinating phenotype. Thus, in the presence of 1 ng/ml, there is a substantial reduction in the extent of myelination, and at 10 ng/ml of TGF-β1 and -β2, there is a complete inhibition of myelination. The inhibitory effect of TGF-β1 appears to require its presence during the period of active myelination; pretreatment of Schwann cells with TGF-β1, or treatment with TGF-β1 after myelin segments had already formed, appeared to have minimal effects on myelination. This inhibition is likely to be a direct effect of TGF-β on the Schwann cell rather than an indirect effect on the neuron. This is consistent with the high level expression of TGF-β receptors by myelinating Schwann cells, but not by neurons (see Fig. 8), and the striking effects of TGF-β on Schwann cell proliferation and cell adhesion molecule expression in contrast to any obvious effects on neurons. The mechanism by which TGF-β inhibits myelination by Schwann cells is not yet known. We observed that although no myelin segments form in the treated cocultures, Schwann cells display detectable and particulate staining for MBP (see Fig. 3 *E*). This result suggests that MBP, and perhaps other myelin components, may continue to be synthesized at low levels in the presence of TGF-β, but cannot be assembled into a myelin sheath. Additional studies will be required to clarify this point.

We have also found that TGF-β1 inhibits assembly of the basal lamina. In particular, the basal lamina appeared patchy and thin in cocultures treated with TGF-β in comparison to untreated cultures (see Fig. 4). These findings are also supported by the attenuated laminin staining and reduced amount of laminin detected in the treated cultures. The inhibition of Schwann cell proliferation by TGF-β with the consequent reduction in the number of Schwann cells may have accentuated this reduction). Of note, the major laminin isoform in these cultures is not laminin-1, but appears to be laminin-2 and/or laminin-4, consistent with previous studies on the expression of these isoforms in peripheral nerve in vivo (Sanes et al., 1990). These effects of TGF-β1 on the Schwann cell basal lamina appear to contrast with its effects on Schwann cells grown alone. Specifically, TGF-β1 has been reported to increase the expression of collagen type IV, but not laminin, mRNAs by adult Schwann cells and to decrease the secretion of tissue-type plasminogen activator and increase the secretion of PAI by adult Schwann cells (Rogister et al., 1993). These results suggest that it promotes extracellular matrix accumulation in purified populations of Schwann cells. Thus, the effect of TGF-β1 on the synthesis of the Schwann cell extracellular matrix may also depend on axonal interactions.

It should be noted that, because TGF-β has pleiotropic effects on Schwann cells, including effects on proliferation and basal lamina production, the inhibition of myelination may be multifactorial. Nevertheless, we were able to distinguish the inhibitory effects of TGF-β1 on Schwann cell proliferation and basal lamina production from those on myelination by seeding excess Schwann cells on neurons and by adding TGF-β1 to the cocultures after a basal lamina had formed, respectively. In addition, the ability of TGF-β1 to antagonize the forskolin-mediated induction of P0 protein (see Fig. 2) and mRNA in Schwann cells maintained under culture conditions that preclude basal lamina formation and proliferation (Morgan et al., 1994), also argues that TGF-β1 can directly inhibit Schwann cell differentiation into the myelinating phenotype.

The Potential Role of TGF-β1 in Development and Degeneration

These studies raise the possibility that TGF-β1 may function as a negative regulator of Schwann cell proliferation and differentiation during peripheral nerve development. Potentially, once an appropriate complement of cells is generated, TGF-β could terminate further proliferation of Schwann cells and/or maintain Schwann cells in an ensheathing state in unmyelinated nerves. We do not favor this model for several reasons. In the studies reported here, rather than finding an increase in the levels of TGF-β in the mature cocultures at a time when Schwann cell proliferation is markedly reduced, we observed the reverse. In addition, anti-TGF-β antibodies that neutralize all three isoforms of TGF-β had no discernible effect on Schwann cell proliferation in the coculture system (Lin, J., and J. Salzer, unpublished observa-

tions), nor did they result in a significant increase in the number of myelinated fibers that formed when Schwann cells were cocultured with either sensory or sympathetic neurons. Rather, we suggest that during development, expression of TGF-β1 is normally downregulated as a result of axonal contact, thereby allowing Schwann cell proliferation and differentiation to progress.

The regulated expression of TGF-β1 and its effects do suggest a role for TGF-β1 during Wallerian degeneration. As noted, TGF-β1 levels increase dramatically after peripheral nerve injury (Rogister et al., 1993; Scherer et al., 1993). In addition, many of the changes that occur in Wallerian degeneration are consistent with the effects of TGF-β on Schwann cells (see also discussion in Scherer et al., 1993). Thus, an increase in TGF-β would be expected to induce proliferation of isolated Schwann cells in the distal stump and increase their expression of NCAM, although perhaps not L1. Also, the increase in TGF-β1 levels that accompany Wallerian degeneration may account for the modest, transient increase in SCIP expression that has been observed several days after nerve transection (Monuki et al., 1990; Scherer et al., 1994). In addition, TGF-β is chemotactic for macrophages (Wahl et al., 1987); its increase during Wallerian degeneration would therefore be expected to promote infiltration of macrophages into the distal stump. Macrophages release interleukin-1 in response to TGF-β (Wahl et al., 1987) which, in turn, has been implicated in the increased expression of NGF and the NGF receptor by nonneuronal cells during Wallerian degeneration (see Brown et al., 1991; Matsuoka et al., 1991). Macrophages may further amplify the response to TGF-β1 by activating latent TGF-β1 (Wahl, 1992), as well as releasing other soluble factors that are mitogenic for Schwann cells (Baichwal et al., 1988). TGF-β1 promotes the survival of motor and sensory neurons (Martinou et al., 1990; Chalazonitis et al., 1992), suggesting it may have neurotrophic activity itself. Finally, TGF-β stimulates the synthesis of tenascin-C by fibroblasts (Pearson et al., 1988; Tucker et al., 1993), an extracellular matrix component that regulates nerve fiber outgrowth and that is known to increase after peripheral nerve injury (Martini et al., 1990).

These effects of TGF-β1 may be important to the ability of nerve fibers to regenerate in the peripheral nervous system. Myelinated nerve fibers (Bedi et al., 1992) and myelin components such as MAG (Mukhopadhyay et al., 1994) are nonpermissive substrates for the regeneration of adult neurons. By recruiting macrophages, which scavenge myelin debris (Stoll et al., 1989; Brown et al., 1991), and by promoting the transition of Schwann cells from a myelinating to a nonmyelinating phenotype, increased TGF-β1 levels would be expected to enhance nerve fiber regeneration in the distal stump. Elevated levels of TGF-β1 might also delay myelination in the distal stump as nerve fibers begin to grow back, thereby preventing the generation of a nonpermissive substrate. Once a full complement of nerve fibers have grown back into the distal stump, TGF-β1 is downregulated (Scherer et al., 1993), presumably allowing subsequent ensheathment and myelination to ensue. By contrast, TGF-β1 does not appear to cause breakdown of myelin that has already formed (data not shown) or to induce the proliferation of Schwann cells already associated with neurites. The apparent absence of effects on mature myelin sheaths may ensure that uninjured fibers proximal to the site of injury, or in close proximity in the case of a partial nerve injury, are not adversely affected by TGF-β.

In summary, we have provided evidence that TGF-β1 has profound effects on axon/Schwann cell interactions that differ from its effects on Schwann cells alone. These data suggest an important role for TGF-β in the regenerative responses that follow peripheral nerve injury, as well as a possible role as a negative regulator of Schwann cell proliferation and differentiation during development.

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Note Added in Proof. A similar inhibitory effect of TGF-β on Schwann cell myelination in vitro was recently reported (Guénard, V., L. A. Gwynn, and P. Wood. 1995. *J. Neurosci.* 15:419–428).

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